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14. ABSTRACT

Breast cancer recurrence is a major clinical obstacle that accounts for greater than 95% of breast cancer mortality in the United States. It is believed to be the result of a subset of breast tumor cells commonly referred to as tumor stem cells that are characterized by broad-spectrum resistance to cytotoxic therapeutics and tumorigenicity. The broad-spectrum resistance to agents that preferentially target proliferating cells is consistent with reports indicating that tumor stem cells are very slow-growing or quiescent cells. Previously we have reported that hyper-activation of the hedgehog signaling pathways subverts quiescence in mammary stem cells. The goal of the current study was to determine if modulation of hedgehog signaling would affect the kinetics of the mammary regenerative cycle and the proliferative activity of mammary stem cells and breast tumor stem cells. Using a combination of genetic and pharmacologic approaches we present data indicating that small molecule modulators of Smoothened are able to bi-directionally regulate hedgehog signalling in vivo and that pharmacologic activation of hedgehog signaling phenocopies the effects of Ptch1 heterozygosity during mammary development and regenerative stasis. Additionally we find that hedgehog activation promotes stem cell proliferation and luminal epithelial cell fate. Finally we present data indicating that taxane resistance can be subverted via pharmacologic activation of hedgehog signaling with SAG1. Together these studies indicate that hedgehog signaling in tumor stem cell populations may subvert quiescence and broad-spectrum chemo-resistance.

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Introduction

In the United States and other countries with access to modern cancer care, breast cancer recurrence accounts for greater than 95% of breast cancer mortality. This highlights the urgent need for the discovery and development of pharmacologic strategies that reduce breast cancer recurrence. Recurrence is widely believed to be the result of a rare sub-population of tumor cells, referred to as tumor stem cells (TSCs) that possess two properties; broad-spectrum resistance to cytotoxic therapeutics and tumor initiating capacity (*1*). ΔNp63α is the predominant product of *TP63* and is required for the long-term preservation of replicative capacity in normal adult stem cells (*2-5*) and is also abundantly expressed in enriched fractions of tumor stem cells. ΔNp63α is an oncogene that represses the tumor suppressive effects of cellular senescence. It is also required for therapeutic resistance in triple-negative breast cancers (*6*). For these reasons we have sought to identify and develop pharmacologic strategies to subvert the activity of ΔNp63α. Previously we have published a study that identified a reciprocal genetic interaction between *TP63* and the hedgehog-signaling pathway that governed cellular quiescence in mammary stem cells (*7*). Quiescence is an important feature of adult stem cells and tumor stem cells because it enables the prolonged retention of proliferative capacity while simultaneously limiting proliferation (*8, 9*). In breast cancer, quiescence is believed to contribute to the broad-spectrum resistance to cytotoxic agents that is a hallmark of tumor stem cells and their ability to initiate disease recurrence. The goal of this proposal was to test the hypothesis that disruption of hedgehog signaling would subvert tumor stem cell quiescence and result in increased sensitivity to cytotoxic agents within tumor stem cell fractions. Additionally the study was designed to determine if aberrant hedgehog signaling and/or subverted mammary stem cell quiescence was a marker of breast cancer pre-malignancy. A combination of mouse genetics, cell biology, in vitro pharmacology and in vivo pharmacology were employed to address these questions. Our results indicate that suppression of hedgehog signaling lead to the execution of cell fate decisions that included the forfeiture of self-renewal and commitment to luminal epithelial differentiation. Consistent with this observation hyper-activation of hedgehog signaling via haploinsufficiency of the negative regulator, Patched 1 (Ptch1) lead to increased elaboration of committed mammary progenitors by mammary stem cells. While technical challenges limited the direct testing of hedgehog inhibitors in vivo, our studies provide proof of principle that suppression of hedgehog signaling subverts quiescence and leads to increased sensitivity to cytotoxic. Additionally hyper-activation of hedgehog signaling in the context of the MMTV-myc breast cancer model had an activating effect suggesting role for hedgehog activation in breast cancer premalignancy. Additional studies will be necessary to determine if hedgehog-signaling status confers increased sensitivity to cytotoxic therapeutics.

Body

Results from Individual Tasks in the Approved Statement of Work

Task 1: Determine the effects of pharmacologic regulation of Smoothened on mammary stem cell quiescence.

Sub-Task 1: Evaluate the effects of Smo agonists on regulation of quiescence in Lin-/CD24+ /CD29high cells.

Sub-Task 2: Evaluate the ability of Smo antagonists to rescue the Ptch1-/+ phenotype.

To successfully complete sub-tasks 1 and 2 we first evaluated the treatment model using the mouse Ptch1$^{tm1Mps/J}$ from The Jackson Laboratory (Stock#003081). Mice were administered with Smo modulators by oral gavage. Treatment doses were set at 15mg/kg for the Smo-agonist SAG and 100mg/kg for the Smo-antagonist SANT2 (*10*). 48h after a single dose, whole mammary glands were harvested and RNA isolated following standard protocols. To evaluate the effect of Smo-modulators on hedgehog pathway activity in mammary tissue, we measured the changes at the mRNA level of target genes of the pathway (Figure 1). We observed increase or decrease in target gene expression after treatment with the Smo-agonist or antagonist respectively, when compare to vehicle alone.

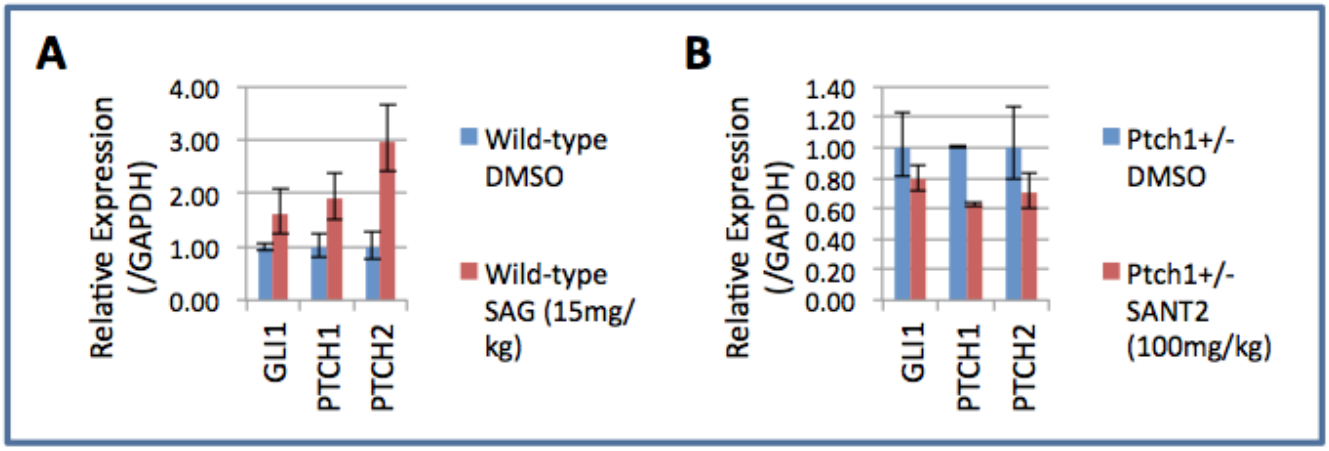

Figure 1. Hedgehog target genes responded *in vivo* to Smo-modulators treatment in mammary glands. A. In vivo treatment of Ptch1 heterozygotes with the Smoothened agonist SAG was sufficient to enhance expression of 3 canonical hedgehog target genes, Gli1, Ptch1 and Ptch2. **B.** in vivo treatment of the Smoothened antagonist SANT2 resulted in decreased expression of Gli1, Ptch1 an Ptch2.

Together these studies indicated that a short-term in vivo treatment model was developed that was sufficient to produce expected patterns of hedgehog signaling modulation. These studies also noted pharmacologic efficacy of SAG that resulted in statistically significant increases in hedgehog target gene expression. Unfortunately while SANT2 did reproducibly repress hedgehog target gene expression statistical significance was achieved for only Ptch2. This difference in efficacy may reflect differences in the in vivo pharmacology of SAG and SANT2 and additional analysis will be necessary to determine the degree to which SANT2 was effectively inhibiting the activity of Smoothened.

After establishing the adequate drug, dose, vehicle and method of administration that efficiently modified the Hedgehog pathway in mammary tissue, we began to evaluate the effect of these treatments on quiescence in mammary stem cells. Previously, we had shown an increase in the ratio of luminal progenitors to mammary stem cells population in the Ptch1+/-, compared to wild-type littermates. This observation is consistent with the hypothesis that abnormal activation of Hedgehog signaling affects the balance between quiescence and mitotic activation in mammary stem cells. We therefore, isolated MECs from Ptch+/- mice treated in vivo with SANT2 or vehicle alone by generating a single cell suspension from freshly dissected mammary glands. Ptch1+/- mice were treated with a single dose of the drug by oral gavage, and the mammary glands harvested 48h later. Quantification of mammary stem cells ($Lin^-/CD24^+/CD29^{high}$) and mammary progenitors ($Lin^-/CD24^+/CD29^{low}$) (*11*), which were separated by FACS analysis, was then performed. The ratio of mammary stem cells to mammary progenitors was then compared in both groups of animals (vehicle versus treatment). Results indicate an increase in the ratio of luminal progenitors to mammary stem cells in Ptch1+/- mice treated with SMO antagonist, compared to genotype matched animals treated with vehicle alone (Figure 2). This observation suggested that SANT2 exerted an anti-proliferative effect on mammary stem cells that is consistent with previous studies indicating that hedgehog signaling of enriched fractions of mammary stem cells ($Lin^-/CD24^+/CD29^{high}$) lead to improved mammosphere formation. Additionally the accumulation of ($Lin^-/CD24^+/CD29^{low}$) luminal progenitors in response to SANT2 is consistent with a blockade in luminal differentiation. Together these results support a model in which hedgehog signaling is pro-mitotic in mammary stem cells and pro-differentiation in committed mammary epithelial fractions. They also indicate that SANT2 disrupts hedgehog signaling in both stem cell and committed fractions. This might reveal a dual role for Hedgehog pathway in the maintenance and differentiation of mammary progenitor cells.

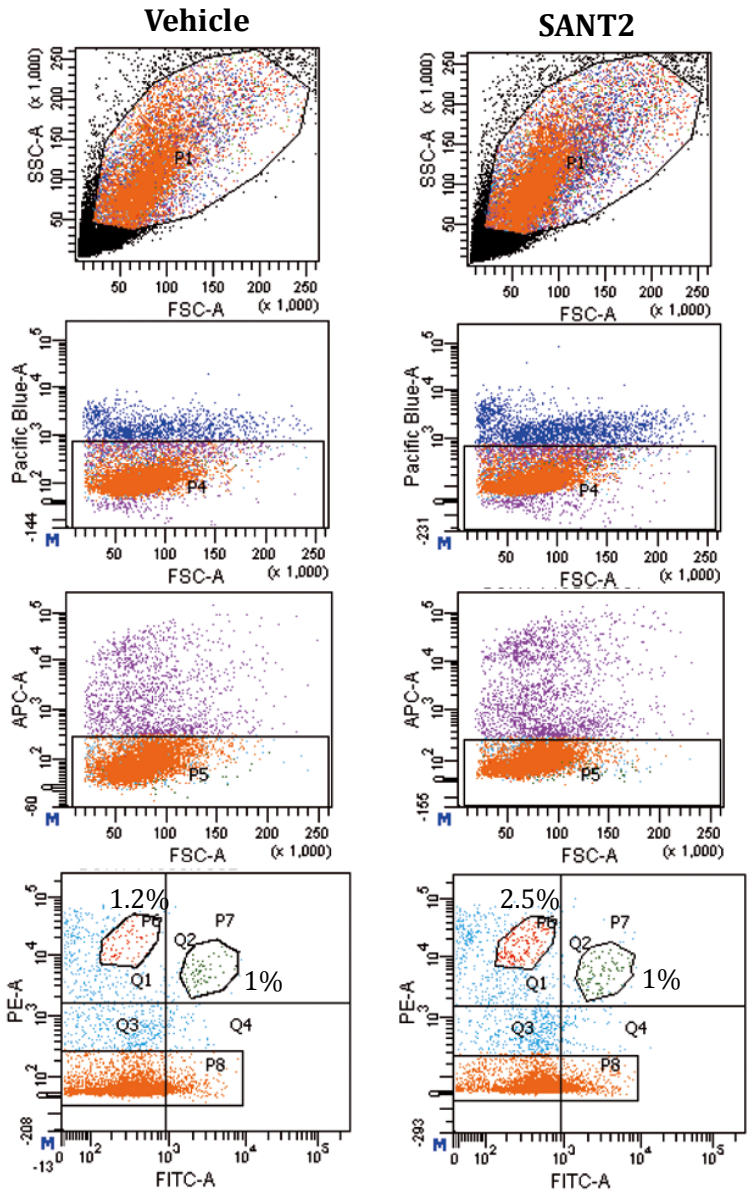

Figure 2. FACS analysis of mammary epithelial cell from Ptch1+/- mice treated with SANT2 or vehicle alone.
Single dose treatment with SANT2 resulted in a two-fold increase in $Lin^-/CD24^+/CD29^{low}$ cells relative to vehicle treated mice (compare P6 from vehicle treated mice to P6 from SANT2 treated mice.

The previous data also suggested that hedgehog signaling promotes luminal epithelial cell fate specification. To confirm this we tested the effects of hedgehog activation on mitotic polarity in the mammary acini-forming assay. Unlike the mammosphere forming assay cells are grown in Matrigel and are able to efficiently form epithelial structures with an exterior layer of CK14-positive basal epithelia and CK19-positive luminal epithelia (*12*). As these acini form expansion of basal epithelium is carried out via symmetric mitosis that is parallel to the outer surface of the sphere. By contrast the elaboration and expansion of luminal epithelia is carried out via asymmetric mitosis that is perpendicular to the outer surface of the structure. To determine if hedgehog signaling affected cell fate IMEC cells were cultured in Matrigel in the presence of recombinant sonic hedgehog with and with out the hedgehog-neutralizing antibody 5E1. Acinar structures were fixed and 2-color immunofluorescence to detect dividing cells

(detected with anti-phospho-HistoneH3) and spindle orientation (detected with tubulin). Mitotic events were counted and scored as parallel or perpendicular to the outer surface of the acinar structure. Results of this study (Figure 3) indicated that addition of hedgehog caused a dramatic shift from parallel to perpendicular mitosis. This finding is consistent with the observation that hedgehog activation promotes luminal cell fate specification.

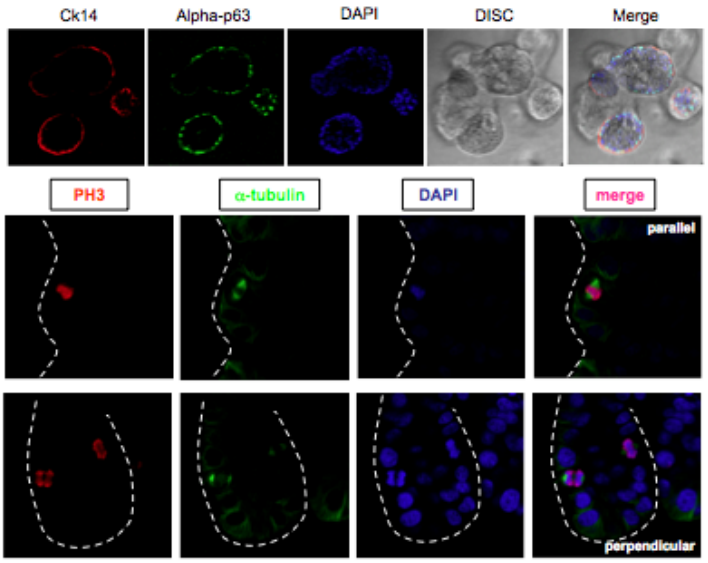

Figure 3: Hedgehog activation promotes asymmetric mitosis and elaboration of luminal epithelia. Upper panel shows immunoflourescent analysis of acinar structures and indicates that they contain diverse cell layers that are either CK14 positive or CK14 negative. Lower panel shows IF analysis of mitotic symmetry in acini -/+ hedgehog signaling. Table 1 shows tabulated data from scoring mitoses as either parallel or perpendicular.

Table 1

	IMECs culture media	Shh-con: IMECs media (1:1)	Shh-con 5E1(1ug/ml)	Shh-con IgG (1ug/ml)
Parallel Division	49.0%+4.4% (n=3)	26.8%+3.4% (n=3) (P<0.05)*	47.1%+2.5% (n=3)(P<0.01)**	32.0%+2.6% (n=3)
Perpendicular Division	51.0%+4.4% (n=3)	73.2%+3.4% (n=3) (P<0.05)	52.9%+2.5% (n=3)(P<0.01)	68.0%+2.6% (n=3)

Preliminary data in support of this proposal indicated that transplantation of mammary stem cells from Ptch1 heterozygotes resulted in improved engraftment efficiency and increased side branching. These studies predicted that prolonged administration of Smoothened agonists would mimic the effects of Ptch1 heterozygosity resulting in enhanced ductal outgrowth and aberrant side-branching. To address this, enriched fractions of mammary stem cells ($Lin^-/CD24^+/CD29^{high}$) were isolated from $C57B/6^{Rosa/+}$ and transplanted into the cleared fat-pads of syngeneic recipients. At day 2 post transplant $C57B/6^{Rosa/+}$ recipients were randomized and treated with vehicle or SAG1 at 30 mg/kg every 48 hours for 20 days. Following the final treatment mammary glands were harvested and evaluated by whole-mount preparation and LacZ staining. Results indicated that while there was no observable effect of SAG1 on engraftment efficiency the ductal outgrowths from the SAG1-treated cohort had significantly greater side-branching and precocious lobulo-alveloar formation (Figure 4).

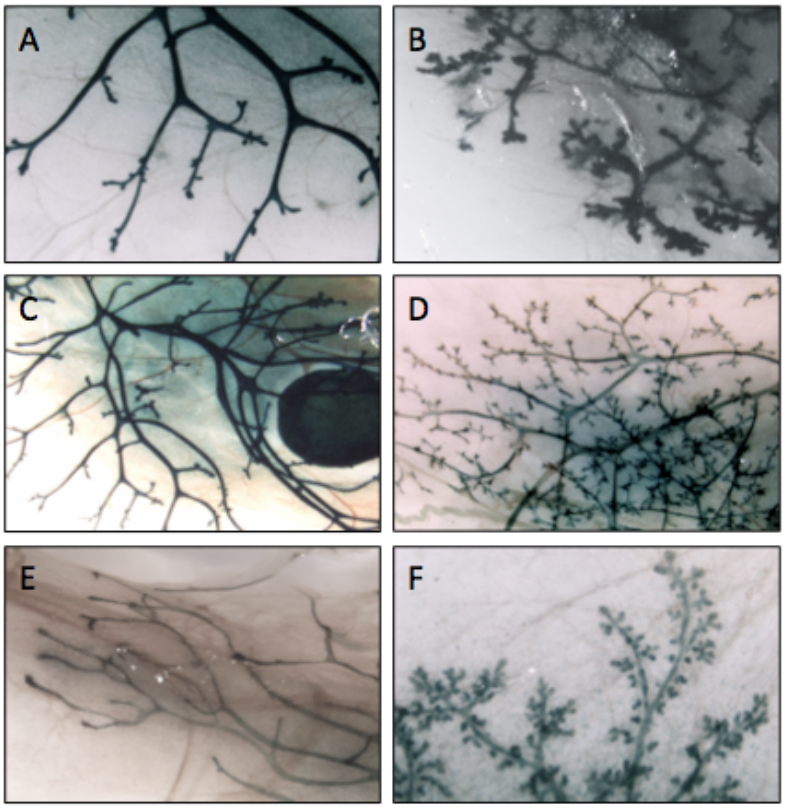

Figure 4: Pharmacologic activation of hedgehog signaling with SAG1 causes aberrant side-branching and precocious lobuloalveolar development. A, C, and E: Represenatative images of LacZ stained whole mounts of ductal outgrowths from recipients treated with vehicle. **B, D and F:** Representative images of LacZ stained whole-mounts of ductal outgrowths from recipients treated with SAG1.

The aberrant side-branching and precocious lobuloalveolar formation in the mammary glands of Ptch1 heterozygotes predicted that disruption of hedgehog signaling with SANT2 would block this phenotype. To test this enriched fractions of mammary stem cells (Lin^- /$CD24^+$/$CD29^{high}$) from $Ptch1^{+/LacZ}$ mice were transplanted into the cleared fat pads of syngeneic recipients (C57B/6 x 129 F1). At day 2 post-transplant, $Ptch1^{+/LacZ}$ recipients were randomized and treated with vehicle or SANT2 at 50 mg/kg every 48 hours for 20 days. Following the final treatment mammary glands were harvested and evaluated by whole-mount preparation and LacZ staining. During the course of treatment mice receiving SANT2 experienced unacceptable toxicities and the experiment was halted at day 6 post-transplant. Whole mount analysis at this point failed to show any ductal outgrowths in either vehicle or SANT2 treated mice, suggesting that the degree of ductal outgrowth at day 6 was insufficient to see by LacZ staining. SANT2-treated mice were feeble, did not eat or drink efficiently and developed peritonitis. At present it is unknown if this toxicity was mediated by inhibition of hedgehog or a more generalized toxicity. Based on this unexpected outcome no conclusions can be drawn regarding the ability of Smoothened antagonists to rescue the mammary gland phenotype observed in the $Ptch1^{+/LacZ}$ mouse.

Conclusions from Task 1

1. Pharmacologic modulation of Smoothened activity, and by extension hedgehog signaling was achievable in vivo.

2. Hedgehog signaling was sufficient to subvert mammary stem cell quiescence which is consistent with the previous finding that Ptch1 heterozygotes have diminished label-retaining capacity.
3. Pharmacologic disruption of Smoothened is anti-mitotic in mammary stem cells and blocks differentiation in committed mammary progenitors.
4. Activation of hedgehog signaling with SAG1 phenocopies the aberrant side-branching and precocious lobuloalveolar development observed in the Ptch1$^{+/LacZ}$ mouse.

Task 2: Determine if hedgehog-mediated defective quiescence predisposes nulliparous MMTV-myc mice to tumorigenesis.

Sub-task 1: Genetic analysis:

The MMTV-myc mouse model of breast cancer is unique in that parity causes a dramatic increase in tumor formation and decrease in latency relative to age-matched nulliparous mice (*13*). Based upon this, we and others have adopted the nulliparous MMTV-myc mouse as a model of pre-malignancy. In this study we hypothesize that mammary stem cells of nulliparous MMTV-myc mice are in a quiescent state that restricts tumor formation and that pregnancy-induced subversion of quiescence in the context of ectopic c-myc results in rapid and highly penetrant tumorigenesis. Previously we have shown that the mammary stem cells in Ptch1$^{+/LacZ}$ were unable to retain BrdU for prolonged periods of time, indicating a defect in quiescence (*7*). To determine if defective quiescence was a feature of premalignancy we conducted genetic crosses of C57B/6x129 Ptch1$^{+/LacZ}$ females with FVB/N MMTV-myc males and genotyped the F1 females to determine Ptch1 homozygosity vs heterozygosity and MMTV-myc positivity. Genotyped mice were segregated into four possible groups:

1. Ptch$^{+/+}$ and MMTV-myc negative
2. Ptch$^{+/+}$ and MMTV-myc positive
3. Ptch$^{+/LacZ}$ and MMTV-myc negative
4. Ptch$^{+/LacZ}$ and MMTV-myc positive

A total of 16 female mice per genotype were identified in this experiment and housed for 12 until the age of 14 months. During that time mice were examined twice weekly; once by the PI (Dr. DiRenzo) and once by the staff in the animal resource center. During this period we observed the following results:

Ptch1 Status	MMTV-Myc Status	Tumors	Average Latency Period
+/+	Negative	0/16	NA
+/+	Positive	11/16 (68.75%)	321.4 +/- 50.8 days
+/LacZ	Negative	0/14*	NA
+/LacZ	Positive	9/13** (69.23%)	314.75 +/- 55 days

* Two of the Ptch$^{+/Lacz}$/MMTV-myc^{-} mice were euthanized prior to the completion of the 14 month period because they were had illnesses unrelated to breast cancer.

** Three of the Ptch$^{+/Lacz}$/MMTV-myc^{+} mice were euthanized prior to the completion of the 14 month period because they were had illnesses unrelated to breast cancer.

These results indicated that Ptach1 heterozygosity did not confer a statistically significant difference in tumor formation between on the MMTV-myc background. We did note a slight reduction in overall latency period however within the Ptch1 heterozygotes however this data

included one Ptch1-heterozygote who developed a tumor at 44 days. We view this very short induction within a single mouse as an outlier. If this induction data is included the average latency period for the Pthc1$^{+/LacZ}$/MMTV-myc^{+} mouse is 284.7 days. If this outlier is dismissed from the data set the average latency period for the remaining 8 mice is 314.75 days, which is not statistically significant. Our rationale for dismissing the 44-day point as an outlier comes from analysis of the standard deviations of the data set with and without that data point. If that point was included the standard deviation is 103.9 days. If that data point was excluded the standard deviation was 55 days. By comparison, the standard deviation of the Ptch1$^{+/+}$/MMTV-myc^{+} data set was 50.8 days. Given the overall rate of tumorigenesis of 68.75% and 69.23% between Ptch$^{+/+}$/MMTV-myc^{+} and Ptch1$^{+/LacZ}$/MMTV-myc^{+} there is greater confidence in the data if the 44-day time point was excluded from statistical analysis of the study.

Based upon this study we conclude that no significant genetic interaction exists between Myc-mediated tumorigenesis and constituative Hedgehog signaling. Given certain flaws in the study, no other conclusion could be drawn. The most significant flaws in the study were:

1. Incomplete tumorigenesis by 14 months.
2. Loss of Ptch1$^{+/LacZ}$ mice due to conditions unrelated to breast cancer or any cancer.

Sub-task 2: "Pseudo-genetic model".
The goals of this study were:

1. To isolate enriched fractions of mammary stem cells (Lin^{-}/CD24^{+}/CD29^{high}) from MMTV-myc mice and suppress Ptch1 expression via lentiviral shRNA and determine if increased tumorigenesis was achieved.
2. To isolate enriched fractions of mammary stem cells (Lin^{-}/CD24^{+}/CD29^{high}) from Ptch1$^{+/LacZ}$ mice and over-express c-myc via lentiviral infection and determine if increased tumorigenesis was achieved.

In these studies Lin^{-}/CD24^{+}/CD29^{high} cells were isolated from MMTV-myc mice on three separate occasions and from Ptch1$^{+/LacZ}$ on two separate occasions. In each case cells were infected with ten times the lentiviral titer necessary to infect IMEC cells to approximately 100%. Infections were carried out using the polybrene/centrifugation method (*14*) that has been previously described for high-efficiency infection of mammary stem cells. Unfortunately in all cases the infection efficiency based upon a GFP control in the identical lentiviral background was less that 2%. Consistent with this we did not note any reduction in Ptch1 mRNA levels despite the fact that the Ptch1 shRNA chosen for the study had been validated. Despite having contacted multiple colleagues in the field, we were unable to overcome these technical obstacles.

<u>Conclusions from Task 2:</u>

1. Constitutive activation of hedgehog signaling via Ptch1 heterozygosity had no statistically significant effect on rates of tumorigenicity in the MMTV-myc model.
2. Constitutive activation of hedgehog signaling via Ptch1 heterozygosity had no statistically significant effect on the average latency period in the MMTV-myc model.

Task 3: Evaluate the ability of Hedgehog activation to sensitize tumor stem cells to taxanes *in vitro*.
Sub-Task 1: Isolate Lin-/CD24+/CD29high from wt and Ptch1-/+ mice and evaluate the effects of paclitaxel on mammosphere formation, and evaluate the ability of Smo antagonist to protect cells from paclitaxel.
Sub-Task2: Isolate Lin-/CD24+/CD29high from wt mice and evaluate the ability of Smoothened agonists to sensitize cells to paclitaxel.

To evaluate the potential of Hedgehog pathway activation to sensitize tumor stem cells we also used an *in vitro* model, the mouse mammary epithelial cells HC11. This model allowed us to optimize drug doses and treatment time points for induction of Hedgehog signaling. HC11 cell lines, were cultured in RPMI 1640 containing 10% heat inactivated FBS, 5 mg/ml bovine insulin and 10 ng/ml murine EGF, and treated with several doses of the Smo-agonist SAG and Smo-antagonist SANT2 in low serum media for 48h before harvesting the RNA. We then evaluated the effect of these treatments by measuring Hedgehog target gene expression (Figure 3). The optimum dose was also further validated (Figure 4). Thus, we have now modulated Hedgehog signaling in mammary glands *in vivo,* and in mammary stem cells *in vitro*.

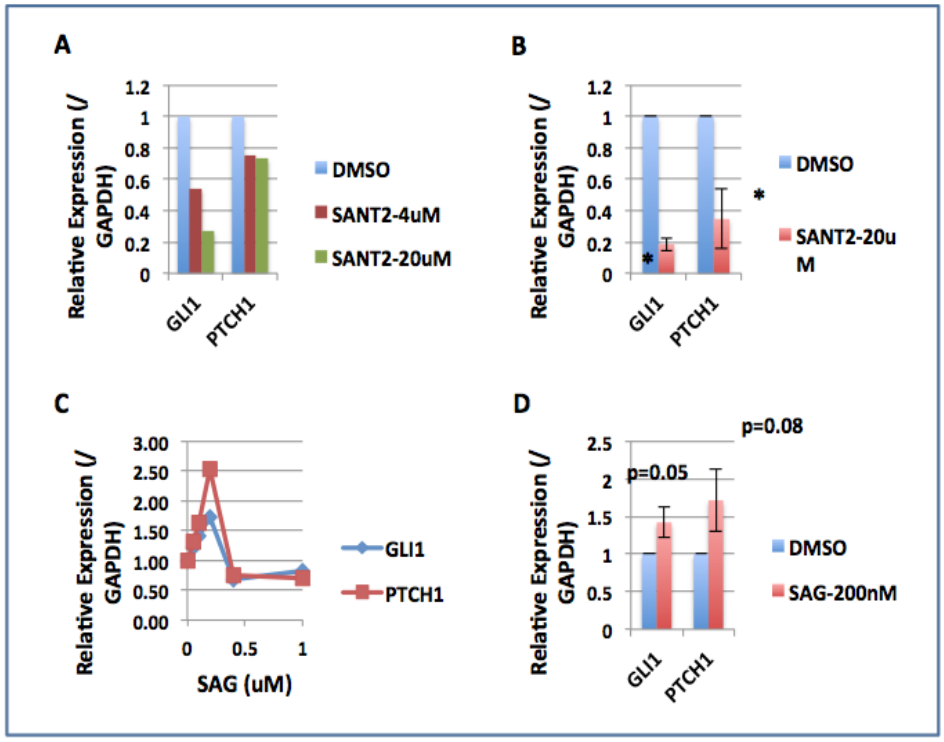

Figure 4: Pharmacologic modulation of hedgehog signaling in the HC11 immortalized mammary epithelial cell line. Relative expression of hedgehog target genes was compared between HC11 cell lines treated with SANT2 (A, B) or SAG (C, D) and vehicle alone. Cells were treated for 48h before RNA was harvested. A representative experiment is shown (A, C). Error bars: Standard deviation in three technical replicates. Asterisk demote statistical significance ($p<0.05$), otherwise p-values are shown.

After establishing the appropriate dose to modulate Hedgehog signaling we tested whether or not activation of this pathway can sensitize the HC11 cell to paclitaxel. In order to perform this analysis we first evaluated sphere formation capacity of HC11 cells. We observed that this cell line can successfully form spheres (>100um) after two weeks culture in low attachment conditions in EpiCult®-B Basal Medium, EpiCult®-B Proliferation Supplements, 10ng/mL EGF, 10ng/mL FGF, and 4ug/mL Heparin. Then we treated the cells with SAG (200nM) or DMSO

and paclitaxel (2.5nM) for 48h in low attachment conditions and remove drugs. After two weeks we quantified the spheres present in the culture and compared the number of total spheres observed in paclitaxel treated cells with cells co-treated with SAG. Our preliminary results (Figure 5) show a dramatic reduction of the number of spheres when cells were also treated with SAG. We are currently working on confirming this results not only in HC11 cells, but also in Lin-/CD24+/CD29high cells derived from wild-type mice, to demonstrate that hedgehog pathway activation through Smo-agonists sensitize cells to taxanes. Being able to confirm these promising results would open a new option for the treatment of breast cancer patients, especially for those with recurrent tumor growths that have a more challenging therapeutic management and worse prognosis.

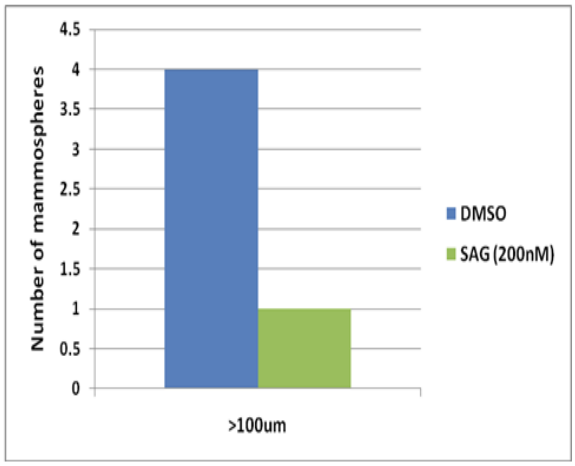

Figure 5: SAG treatment sensitizes mammary tumor-sphere forming cells to taxol. HC11 cells were treated with DMSO control or SAG plus taxol for 2 days in under low attachment conditions and then drugs were removed and cell were cultured for 14 days. Multicellular structures greater than 100 microns were scored as mammary spheres. Results indicate that SAG treatment enhanced the ability of taxol to suppress sphere-forming cells.

While this result is preliminary it does support is consistent with the hypothesis that stimulation of mammary stem cells with hedgehog activators subverts quiescence, thereby sensitizing cells to cytotoxic agents such as taxol.

Conclusions from Task 3

1. Hedgehog signaling can be positively or negatively modulated regulated with SAG1 or SANT2 in HC11 cells.
2. Under conditions in which SAG1 clearly induces hedgehog signaling it is able to sensitize sphere-forming cells to taxol.

Key Research Accomplishments

1. Pharmacologic modulation of Smoothened activity, and by extension hedgehog signaling was achievable in vivo. In this study we demonstrated that in vivo administration of the Smoothened Agonist, SAG1, or the Smoothened Antoagonist (SANT2) elicited measurable effects on expression of hedgehog gene expression.
2. Hedgehog signaling was sufficient to subvert mammary stem cell quiescence which is consistent with the previous finding that Ptch1 heterozygotes have diminished label-retaining capacity. In this study we noted that in vivo treatment with SAG resulted in increased numbers of committed mammary progenitors which is consistent with increase elaboration of progenitors from mammary stem cells via asymmetric mitosis.

3. Pharmacologic disruption of Smoothened is anti-mitotic in mammary stem cells and blocks differentiation in committed mammary progenitors. In this study we demonstrate that activation of hedgehog signaling causes a dramatic shift in mitotic spindle polarity that is consistent with luminal epithelial elaboration.

4. Activation of hedgehog signaling with SAG1 phenocopies the aberrant side-branching and precocious lobuloalveolar development observed in the $Ptch1^{+/LacZ}$ mouse. In this study, we observed that SAG treatment during mammary ductal outgrowth resulted in a recapitulation of the aberrant side-branching and precocious lobuloalveolar development that was observed in the $Ptch1^{+/LacZ}$. These studies indicate that the hedgehog modulators used in these studies are biologically active in vivo.

5. Activation of hedgehog signaling had no statistically significant effect on c-myc-mediated mammary tumorigenesis or latency period.

6. Activation of hedgehog signaling with SAG1 sensitizes the sphere-forming fraction of cells to taxol in vitro. In this study we demonstrate that hedgehog activation is sufficient to increase sensitivity to cytotoxic therapeutics. This is consistent with the finding that hedgehog activation is sufficient to subvert stem cell quiescence.

Reportable Outcomes

Presentation of a poster by James DiRenzo Ph.D. describing aspects of this work were made at the DoD Era of Hope Conference in Orlando Florida in 2011.

Presentation of a poster by Avencia Mejias Ph.D. and David J. Robbins Ph.D. describing aspects of this work were made at the DoD Era of Hope Conference in Orlando Florida in 2011.

Conclusion

From the studies completed with the support of this award we draw the following conclusions.

1. Hedgehog signaling can be targeted in vivo with small molecule modulators of Smoothened activity.
2. Doing so results in predictable changes in hedgehog target gene expression in vivo.
3. Activation of hedgehog signaling during mammary ductal outgrowth with SAG1 recapitulates the $Ptch1^{+/LacZ}$ phenotype.
4. A single SANT1 treatment in vivo was sufficient to increase the committed luminal progenitor population by 2 fold. This result likely highlights the requirement for hedgehog to complete the luminal epithelial program of differentiation.
5. Activation of hedgehog signaling with SAG1 sensitizes mammosphere-forming cells to taxol.

The overarching hypothesis supporting this proposal held that stem cell quiescence accounted for resistance to cytotoxic therapeutics and that this quiescence could be subverted via activation of hedgehog signaling. The conclusions listed above are consistent with this hypothesis and support the assertion that hedgehog signaling may be targeted for therapeutic benefit in breast cancer. More specifically hedgehog modulators may help to sensitize the so-called tumor stem cells within a breast tumor by forcing them back into the cell cycle. Doing so in the context of adjuvant therapeutics is likely to result in more durable responses to breast cancer therapy.

Previously we have shown that activation of hedgehog signaling via Ptch1 heterozygosity was sufficient to subvert mammary stem cell quiescence (*7*). Based upon this finding we sought to determine if subversion of mammary stem cell quiescence might be an early event in breast tumor initiation. To do this we chose to genetically cross, constitutive hedgehog signaling into the MMTV-myc model, reasoning that if stem cell quiescence accounted for the very low penetrance of tumors in nulliparous. Our results here indicate that activation of hedgehog signaling had no effect on c-myc-mediated tumorigenesis or on latency periods. This may suggest that stem cell quiescence is irrelevant to tumorigenesis or that it was simply not relevant in the MMTV-myc model. Additional studies will be necessary to determine if the effects of hedgehog signaling on mammary stem cell quiescence contribute to breast tumor initiation.

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